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Activation of the NF-κB and Iκ B System in Smooth Muscle Cells after Rat Arterial Injury

Induction of Vascular Cell Adhesion Molecule-1 and Monocyte Chemoattractant Protein-1

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The NF-κB transcription factor family and its inhibitory proteins (IκB) form an autoregulatory system that has been linked to endothelial gene expression and vascular disease. To determine the role of the NF-κB/IκB system in smooth muscle cells (SMCs) *in vivo*, the present study used the balloon catheter injury model in the rat carotid artery. The NF-κB family members p50, p65, p52, c-Rel, and RelB as well as the inhibitor proteins IκBα, IκBβ, and p105 were present in uninjured arteries as determined by immunoblotting. Using electromobility shift assays, low levels of constitutively activated p50, p65, and c-Rel were seen in normal carotid arteries and a fivefold induction occurred during times of rapid SMC proliferation and neointima formation after balloon denudation. Furthermore, immediately after injury, the levels of IκBα, IκBβ, and p105 were dramatically reduced. Expression of the NF-κB-regulated genes, vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic protein (MCP)-1, was apparent in SMCs within 4 hours after injury. Macrophage infiltration occurred in parallel with the expression of VCAM-1 and MCP-1, and these inflammatory cells were present on the luminal surface of injured vessels during intimal lesion formation. In chronically denuded vessels, the SMCs on the luminal surface continued to express high levels of VCAM-1 and MCP-1, which may account for the increased presence of macrophages. Together, these findings link the activation of NF-κB to intimal lesion formation and to the inflammatory response associated with SMCs after vascular injury. ***(Am J Pathol 1997, 151:1085–1095)***

The NF-κB/Rel family of transcription factors (NF-κB) is expressed in a variety of cell types and plays a crucial role in regulating genes that control cell growth, differentiation, and cell death pathways.[1–4] NF-κB exists as a dimeric complex and transmits signals to the transcriptional machinery in the nucleus by binding to κB sites present in *cis*-acting regulatory regions of DNA. The NF-κB family shares the Rel homology domain and includes the following: p50,[105] p52,[100] p65, c-Rel, and RelB. The subcellular localization, binding properties, and transcriptional properties of NF-κB are regulated by a group of inhibitory proteins that include IκBα, IκBβ, IκBγ, bcl-3, p105, and p100.[5] After an activating signal, IκB is phosphorylated and then degraded, allowing the translocation of NF-κB to the nucleus where it can function. In addition, NF-κB has been implicated in the transcriptional regulation of IκBα.[6] Therefore, NF-κB and IκB activity is thought to be regulated via a feedback mechanism referred to as the NF-κB/IκB system.[7,8] This system has been studied extensively in endothelial cells, but little is known about the role of NF-κB in vascular smooth muscle cells (SMCs) and even less is known about the role of this transcription factor in SMCs *in vivo*.

It has been shown that a low level of constitutive NF-κB activity is present in cultured vascular SMCs and that NF-κB is further activated using a variety of stimuli, ie, thrombin, interleukin (IL)-1β, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (FGF-2).[9–12] Several lines of evidence suggest that NF-κB plays an important regulatory role in SMCs. First, Bellas et al reported that NF-κB activity is essential for proliferation of cultured bovine vascular SMCs.[13] Second, Shin et al demonstrated that vascular cell adhesion molecule (VCAM)-1 gene expression is NF-κB dependent in human SMCs.[14] And third, NF-κB has been implicated in atherosclerosis as activated NF-κB is present in human atherosclerotic lesions.[15] Together, these data indicate that NF-κB plays an important role in regulating SMC gene expression and proliferation and perhaps during the pathogenesis of vascular disease.

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Recently, we reported that the NF-κB/IκB system is activated in vascular endothelial cells *in vivo* after balloon catheter injury in rats[16] in conjunction with endothelial expression of VCAM-1 and adhesion of monocytes and macrophages. VCAM-1 gene expression is NF-κB dependent in endothelial cells,[17] and our study showed that there is a correlative link between the activation of the NF-κB/IκB system, NF-κB-dependent gene expression, and vascular inflammation *in vivo*. In the present study, we identify the components of the NF-κB/IκB system in SMCs *in vivo* and show activation of NF-κB after vascular injury. We also demonstrate that the activation of this system correlates with SMC proliferation and with the induced expression of the NF-κB-dependent genes VCAM-1 and monocyte chemoattractant protein (MCP)-1. These two genes are likely to play a role in the resulting macrophage infiltration. Our findings suggest that a link exists between induction of the NF-κB/IκB system and SMC growth and activation after vascular injury.

Materials and Methods

Arterial Injury Model

All animal studies were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (400 g; 3 to 4 months old) were purchased from Taconic, Germantown, NY. All surgical procedures were carried out under general anesthesia by intraperitoneal injection of xylazine (2.2 mg/kg; AnaSed, Lloyd Laboratories, Shenandoah, IA) and ketamine (50 mg/kg body weight; Ketaset, Aveco Co., Fort Dodge, IA). The left and right carotid artery and the aorta were denuded with a 2F balloon catheter as recently described.[18] De-endothelialized segments of arteries were identified by intravenous injection of Evans blue (0.3 ml of 5% solution in saline) 10 minutes before sacrifice. Animals were perfusion-fixed with phosphate (0.1 mol/L, pH 7.4) buffered 4% paraformaldehyde for *in situ* hybridization and with methanol (80%) buffered with Tris/saline (130 mmol/L NaCl, 25 mmol/L Tris, pH 7.6) for immunostaining. For Western and Northern blotting, animals were perfused with ice-cold lactated Ringer's solution to remove blood and plasma proteins followed by excision and removal of adventitial tissue. The vessels were then snap-frozen in liquid nitrogen. Rats were sacrificed at the indicated times after injury.

Northern Blot Analysis

Common carotid arteries were harvested from normal rats as well as from rats 6 hours and 3, 7, and 28 days after balloon injury (three to four animals per time point). Vessels were stripped of periadventitial fat and connective tissue in phosphate-buffered saline at 4°C and were then snap-frozen in liquid nitrogen. Frozen arterial tissue was ground to a fine powder under liquid nitrogen, and total cellular RNA was prepared by acid guanidinium thiocyanate extraction.[19] Agarose gel electrophoresis of RNA (15 μg of total RNA per lane and transfer to nylon membranes (Zeta Probe, Bio-Rad Laboratories, Richmond, CA) were carried out as previously described.[20] After transfer, RNA blots were exposed to shortwave ultraviolet light both to cross-link RNA to the membrane and to visualize the major ribosomal RNA bands. The blot was hybridized using cDNA probes labeled with [^{32}P]dCTP by random primer extension (Amersham, Arlington Heights, IL), washed at 60°C in two changes of 0.045 mol/L NaCl/0.0045 mol/L sodium citrate, pH 7.0/0.1% SDS for 10 minutes each, and then exposed to Kodak X-AR5 film at −70°C.

Western Blotting

Left and right common carotid arteries were harvested from rats at the indicated time points after balloon injury to prepare vessel wall extract samples. The endothelium was removed from uninjured carotid arteries by gently scraping the luminal surface with a cotton applicator immediately before snap freezing. Two independent sets of samples were analyzed (two times two rats per time point), and representative immunoblots are shown. The vessels were ground up in liquid nitrogen using mortar and pestle, and the resulting powder was resuspended in buffer C[21] containing 20 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1.5 μg/ml pepstatin A, 40 μmol/L calpain inhibitor I (Boehringer Mannheim, Indianapolis, IN), 1 mmol/L Na_3VO_4, and 1 mmol/L NaF. A protein extract was generated by freeze-thawing the suspension three times and then removing the insoluble material by centrifuging at 14,000 rpm for 10 minutes at 4°C. The protein concentrations were determined by the Coomassie protein assay (Pierce, Rockford, IL). Twenty micrograms of extract per lane was loaded onto SDS-polyacrylamide gels (10%) followed by electrophoresis. Prestained molecular weight standards (Bio-Rad) were run on each gel. Proteins were electroblotted onto nitrocellulose (BA 85, Schleicher and Schuell, Keene, NH) and stained with primary antibodies and a horseradish-peroxidase-coupled secondary antibody and detected with the ECL detection system (ECL, Amersham Corp.). Bands were visualized on film and the intensity quantitated by densitometry using a scanner and image analysis software (NIH Image 1.60) for Macintosh computers. Equal protein loading of the samples was further verified by evaluation of Coomassie-stained gels and staining of blots with antibodies against α-smooth muscle actin (Sigma 1A4, Sigma Chemical Co., St. Louis, MO) and the transcription factor Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) (data not shown).

cDNA Probes and Antibodies

For *in situ* hybridization and Northern blotting, the following probes were used. We cloned a 364-bp fragment from the coding region of rat MCP-1 into pCRII (Invitrogen Corp., San Diego, CA) by reverse transcriptase polymerase chain reaction from rat vessel wall mRNA with primers based on the published sequence.[22] The identity of the clone was verified by sequencing. We obtained the cDNAs for rat VCAM-1,[23] murine p50 (kindly provided by Dr. Michael Leonardo, National Institute of Allergy and Infectious Diseases, National Institutes of Health), murine p65 (kindly provided by Dr. David Baltimore, Massachusetts Institute of Technology),[24] and rat IκBα (kindly provided by Dr. Rebecca Taub, University of Pennsylvania School of Medicine).[25] A murine IκBβ cDNA was kindly provided by Dr. Sankar Ghosh (Yale University School of Medicine), and the murine cDNA for c-Rel was kindly donated by Dr. Ranjan Sen (Brandeis University). For synthesis of sense and antisense riboprobes, a 0.46-kb *Eco*RI-*Sac*I fragment of IκBα coding region and a 1.5-kb *Pst*I fragment of the p50 cDNA was subcloned into a transcription vector (pBluescript, Stratagene, La Jolla, CA). A 1090-bp *Xho*I fragment of the c-Rel cDNA was subcloned into pBluescript. Antibodies directed against the following proteins were purchased from Santa Cruz Biotechnology: p50, p65, c-Rel, RelB, p52, bcl-3, IκBα, IκBβ, IκBγ, and VCAM-1. Antibodies directed against rat monocyte/macrophages (ED-1), rat T and B cells (CD5 and OX19), and rat leukocytes (CD45 antigen and OX1) were purchased from Serotec (Indianapolis, IN). The antibody against the rat α4 integrin was from PharMingen (San Diego, CA).

In Situ *Hybridization and Immunostaining*

In situ hybridization and immunostaining was carried out on *en face* preparations of vessel segments as recently described[26] with the exception that the incubation with primary antibody was followed by three washes with Tris-buffered saline (75 mmol/L Tris, 0.4 mol/L NaCl, pH 7.4). A mouse monoclonal antibody recognizing rat monocytes/macrophages (ED-1) was used at a 1:200 dilution. All other antibodies were used at a final concentration of 1 μg/ml. Controls for immunostaining were included in all staining runs using matching concentrations of nonimmune IgG. After *in situ* hybridization, the slides were coated with autoradiographic emulsion (Kodak NTB2) and exposed for 3 weeks and then developed (Kodak D-19). Preparations were observed under the light microscope using dark-field and bright-field illumination.

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay

Ten micrograms of whole-cell extract (preparation described above) was tested in a standard EMSA[27] in a final volume of 15 μl with binding buffer containing 10 mmol/L Tris, pH 7.6, 50 mmol/L NaCl, 0.5 mol/L EDTA, pH 8.0, 4% glycerol, 1 μg of poly(dI-dC), and a double stranded ^{32}P-labeled oligonucleotide duplex containing either the VCAM-1 site from the human VCAM-1 promoter 5′-CTGGGTTTCCCCTTGAAGGGATTCCC-3′, an NF-κB consensus site 5′-AGTTGAGGGGACTTTCCCAGGC-3′, or the c-*myc* promoter 5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′. The binding reaction was electrophoresed on a 4% nondenaturing polyacrylamide gel. The specificity of complex formation was tested by adding to the reaction a 50-fold molar excess of double-stranded unlabeled wild-type NF-κB binding DNA, mutant NF-κB binding DNA (5′-AGTTGAGGCGACTTTCCCAGGC-3′), or an irrelevant DNA probe. To characterize the specific complexes, antibody supershifts were performed as previously described[28] using affinity-purified rabbit antisera directed against p50, p52, c-Rel, or RelB; affinity-purified goat anti-p65 antisera; or as a negative control, normal rabbit Ig or normal goat Ig. The Rel antibodies used in these experiments were from Santa Cruz Biotechnology. Briefly, supershifts were performed by incubating 10 to 20 μg of vessel SMC extract with 1 μl of antibody for 15 minutes at room temperature in binding buffer followed by incubation with 20,000 cpm of the radioactive probe for 10 minutes and then further processed as a standard EMSA.

Results

Expression of Rel Family Members in the Vessel Wall after Balloon Injury

To identify the Rel family members in the SMCs of the vessel wall we performed Western blotting with affinity-purified antibodies and quantitated the changes in protein expression after balloon catheter denudation using densitometry. Extracts for immunoblotting were prepared from normal rat carotid arteries and from carotid arteries at various time points after balloon injury. These time points cover the period of maximal SMC proliferation in the media (day 3), the maximal cell replication in the developing neointima (day 7), the intimal lesion with high cell density and decreasing replication (day 14), and the return to quiescence with a mature intimal lesion (day 28) after balloon catheter denudation.[18] This allowed us to correlate the expression levels of the individual Rel proteins with cellular events associated with intimal lesion formation.

Immunoblots stained with an antibody against p50 revealed the presence of p50 protein in normal carotid arteries (Figure 1A). After denudation, two- to threefold higher levels of p50 were present in denuded vessels at 3, 7, and 14 days after injury. The p50 protein content in 4-week-injured vessels was close to that found in normal vessels. p50 mRNA was detectable in neointimal SMCs by *in situ* hybridization using the *en face* technique (data not shown.)

Immunoblot staining with the p65 antibody showed a single band in all vessel samples (Figure 1B). Compared with normal carotid arteries, an approximate threefold increase in p65 protein was seen by 3 and 7 days after injury. At 2 weeks after balloon injury, levels of p65 protein were even higher, approximately four times the value detected in normal vessels, and a decrease to near normal levels was seen by 28 days after denudation. *In situ* hybridization and immunostaining performed on *en face* preparations demonstrated the presence of p65 mRNA and protein in intimal SMCs (data not shown.)

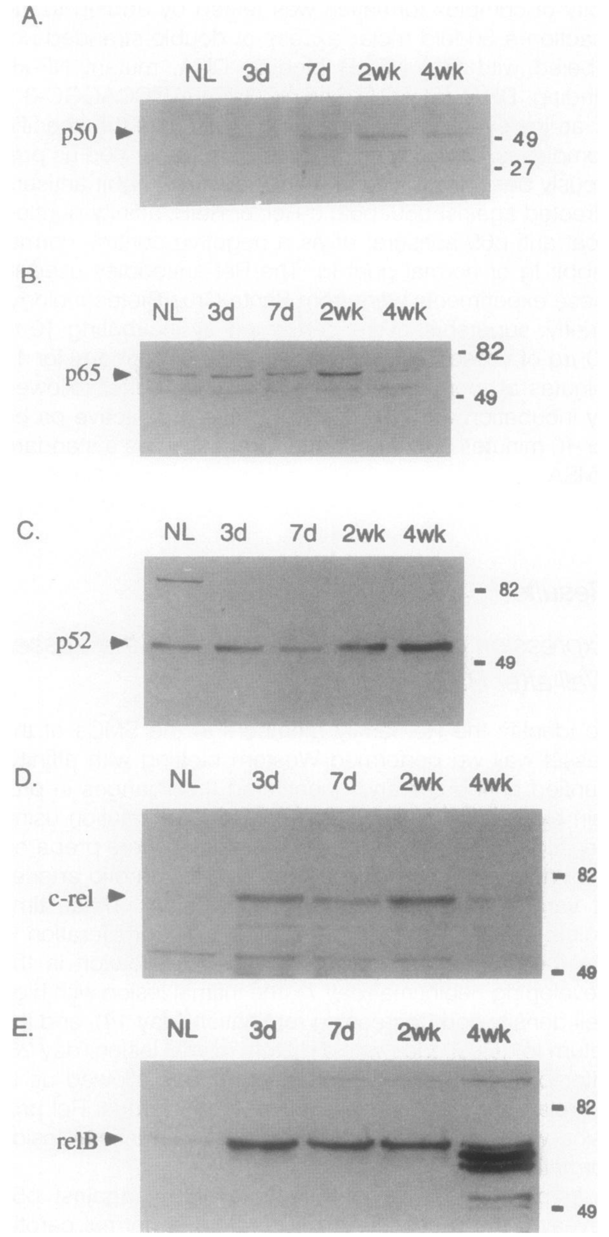

Figure 1. Western blot analysis of the Rel proteins in rat carotid artery extracts at various times after balloon injury. Equal amounts of protein were loaded in each lane. Staining with an antibody against p50 (**A**), p65 (**B**), p52 (**C**), c-Rel (**D**), and RelB (**E**).

Staining the Western blot with an antibody against p52, a band with the appropriate molecular weight was apparent in normal vessel wall extracts (Figure 1C). In addition, a protein with an approximate molecular mass of 100 kd (likely to be the p100 precursor of p52) was detectable in low levels only in the normal vessel wall (Figure 1C). Levels of p52 increased fourfold after balloon injury, and the highest levels were seen 4 weeks after denudation.

Western blots revealed that very low levels of c-Rel protein were present in the normal carotid artery with an apparent molecular mass of 72 kd (Figure 1D). However, this protein was dramatically induced 15- and 10-fold by 3 and 7 days after injury, respectively. Levels of c-Rel peaked at 2 weeks with a 20-fold increase over normal vessels. Four weeks after injury, the level of c-Rel protein declined to five times higher than in normal vessels. c-Rel mRNA was detectable in intimal SMCs by *in situ* hybridization (data not shown).

Using the antibody recognizing RelB, a single band was detected in extracts from normal vessels (approximate molecular mass, 68 kd; Figure 1E). The intensity of this band was seven- to eightfold higher in extracts of vessels harvested 3, 7, and 14 days after injury. At 4 weeks after denudation, this band decreased to normal levels; however, two prominent bands of slightly lower molecular mass became apparent.

Expression of IκB and Inhibitory Proteins in the Vessel Wall after Balloon Injury

The same samples analyzed for Rel protein expression were used to determine the levels of the inhibitor proteins IκBα, IκBβ, IκBγ, and p105 by immunoblotting. The antibodies directed against IκBα and IκBβ each recognized a single band with the expected molecular masses of approximately 37 and 43 kd, respectively. The amount of IκBα protein decreased approximately 55% by 1.5 hours after injury and began to recover by 24 hours, reaching normal levels within 3 to 7 days (Figure 2A). Northern blots probed with a rat IκBα cDNA also showed a similar hybridization signal among these samples (data not shown). *In situ* hybridization carried out on *en face* preparations also demonstrated abundant IκBα mRNA expression by intimal SMCs after injury (Figure 2C).

IκBβ protein is present in normal carotid arteries and decreases immediately after injury approximately 80% and then begins to recover by 24 hours, reaching normal levels within 3 to 7 days (Figure 2A). *In situ* hybridization revealed high expression of IκBβ mRNA in replicating SMCs of the developing neointima at 8 days after injury (Figure 2D).

A Western blot analysis was also performed with an antibody raised against the carboxyl-terminal end of p105, and thus, it is unreactive with p50 but does recognize the inhibitory/precursor protein p105 and the inhibitor IκBγ (an alternatively spliced form of the p105 gene). When the IκBγ antibody was used, no 70-kd IκBγ protein was detectable in rat carotid artery samples, but a protein of approximately 105 kd was present in extracts from the vessel wall. p105 protein was present in normal vessels and decreased approximately 58% within 1.5 hours after injury. The level of p105 protein began to recover by 3 days and returned to normal by 7 days (Figure 2B).

NF-κB Activation

The IκBα, IκBβ, and p105 protein levels decreased in SMCs immediately after injury, which suggested that NF-κB-related proteins become activated in response to injury. Therefore, we examined the whole-cell extracts from normal and balloon-injured rat carotid arteries and aorta for NF-κB binding activity by EMSA using an NF-κB consensus binding site probe and NF-κB binding site probes

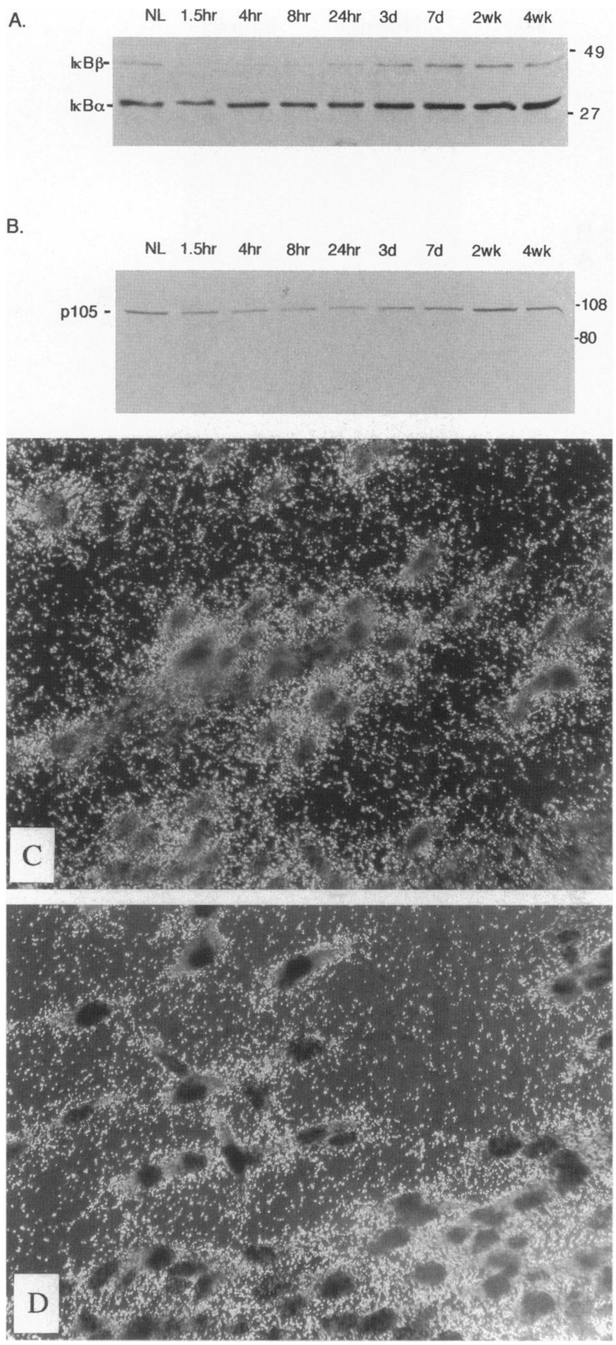

Figure 2. Expression of the NF-κB inhibitors in the rat carotid artery after balloon injury. Immunoblot of vessel wall extracts from normal (NL) carotid arteries and 1.5, 4, 8, and 24 hours, 3 days, 7 days, and 2 weeks after balloon injury are stained with antibodies against IκBα and IκBβ (**A**) and with an antibody recognizing IκBγ and p105 (**B**). **C**: *In situ* hybridization with an antisense riboprobe for IκBα was carried out on an *en face* preparation of a carotid artery 8 days after denudation. The photomicrograph shows specific hybridization (white silver grains) over SMCs present on the luminal surface. **D**: *En face in situ* hybridization for IκBβ 8 days after balloon injury showed a very strong signal over intimal SMCs. **C** and **D**: Specimens are seen under dark-field illumination after nuclear counterstaining with hematoxylin. Original magnification, ×400.

from the VCAM-1 promoter and the c-*myc* promoter. A major complex (complex A, Figure 3A) was detected in SMC extracts from normal carotid arteries and normal aortas (data not shown) using an NF-κB consensus probe, and this binding activity decreased immediately after injury by approximately 80%. By 4 hours after injury, complex A was induced in the carotid artery (Figure 3A) and in the aorta, and a slower migrating complex (complex B) was also induced. By 7 days after injury, complex B was induced approximately fivefold whereas complex A was induced only two- to threefold (Figure 3A). Complexes A and B generated with normal and 3-day-injured carotid extracts competed with a 50-fold molar excess of unlabeled wild-type NF-κB binding probe but not with a 50-fold molar excess of a DNA probe containing a mutation in the NF-κB binding site (Figure 3B) or an excess of an octamer binding probe (data not shown). This demonstrates that the detected complexes represent a specific protein DNA interaction within the NF-κB binding site and suggests that NF-κB-related proteins are present in an active form in normal arteries and are more abundant in injured vessels. A similar NF-κB binding pattern was also detected with DNA probes containing an NF-κB site from the VCAM-1 promoter and from the c-*myc* promoter (data not shown).

The competition data suggested that the complexes generated with SMC extracts from carotid arteries recognize the NF-κB binding site. Therefore, to test whether the complexes consisted of an NF-κB family member, we examined the normal carotid extracts and 3- and 7-day after injury extracts by an antibody supershift assay. Supershifts performed with an NF-κB consensus probe are provided here as this probe generates the lowest background/nonspecific binding. Antibodies directed against p50, p65, and c-Rel supershifted a protein/DNA complex generated with normal, 3-day (Figure 3C) and 7-day extracts (data not shown). Antibodies directed against p52 and Rel B or normal rabbit or goat IgG (Figure 3C) did not generate a supershifted complex. Complex B supershifted with anti-p65 and to a lesser extent with anti-p50 and anti-c-Rel, and complex A supershifted with anti-p50 only. An antibody directed against RelB did not generate a supershifted complex from rat vessels, although this antibody did generate a supershifted complex when tested on rat thymus extracts (data not shown). Together, these data demonstrate that low levels of p50, p65, and c-Rel are constitutively activated in SMCs from the normal vessel wall, and the same subset of NF-κB members are differentially induced in injured arteries.

VCAM-1 and MCP-1 Gene Expression and Adhesion of Inflammatory Cells

NF-κB has been implicated in the induction of VCAM-1 and MCP-1 in endothelial cells and fibroblasts, respectively,[29,30] and both cell types are present in the vessel wall. In addition, SMCs have been reported to express these genes,[31,32] and VCAM-1 induction in SMCs requires the activation of NF-κB.[14] VCAM-1 expression has been reported in rabbit atheroma,[31] and MCP-1 was induced in SMCs after balloon injury.[32] Furthermore, we have previously reported the induction of VCAM-1 expression in wounded endothelium of rat arteries, and

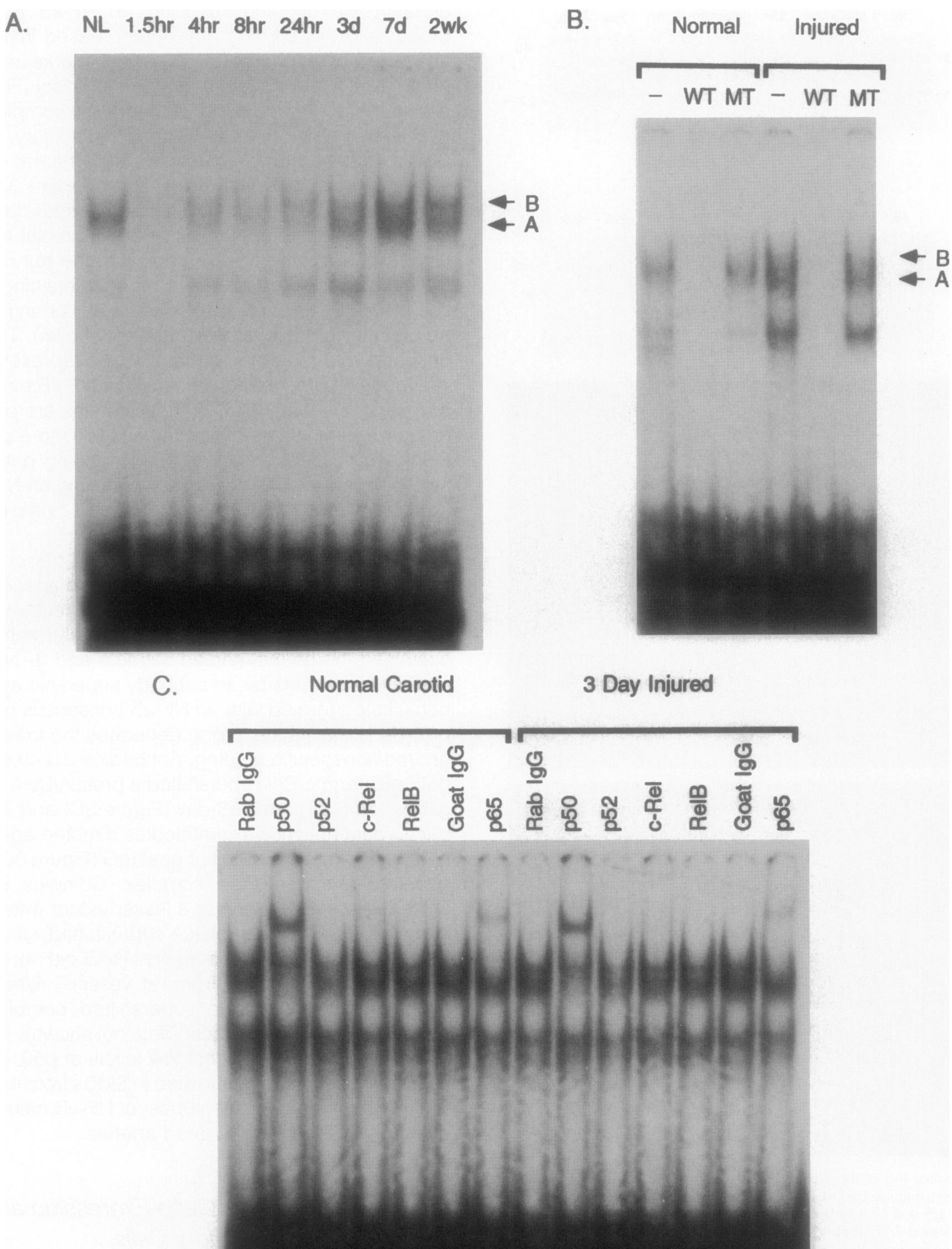

Figure 3. Activated NF-κB is present in normal rat carotid arteries *in vivo* and is induced after injury. **A:** EMSAs were performed using 10 μg of whole-cell extracts from normal (NL) carotid arteries and 1.5, 4, 8, and 24 hours, 3 days, 7 days and 2 weeks after balloon injury and the NF-κB consensus probe. Five separate experiments were performed with a pool of four to six carotid arteries in each sample. Complex A is present in normal carotid whereas complex B is induced after injury. Complexes A and B are indicated with **arrows**. **B:** Competitions were performed with 50-fold molar excess of wild-type (WT) or mutant (MT) NF-κB binding probe using normal or 3-day-injured carotid artery extracts. **C:** Supershifts were performed with 20 μg of normal (NL) artery extract or 10 μg of 3-day-ballooned injured carotid artery extract in addition to one of the following antibodies: normal rabbit IgG (R) or goat IgG (G) or antibodies directed against p50, p52, c-Rel, RelB, or p65. Antibodies directed against p50, p65, and c-Rel supershifted the complexes formed with the NF-κB consensus probe.

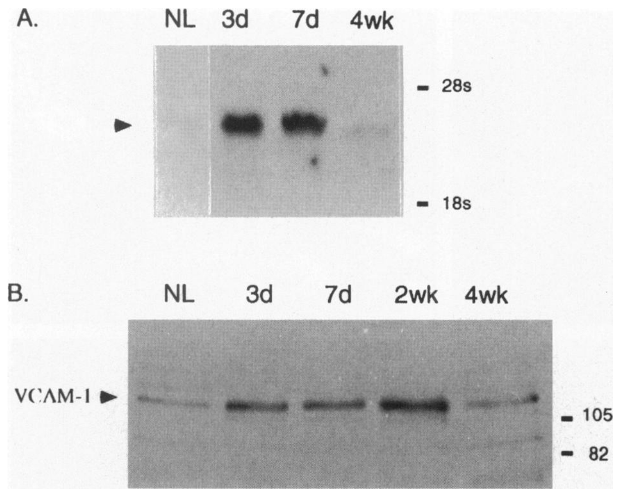

Figure 4. Expression of VCAM-1 in the rat carotid artery after balloon injury. **A:** Northern blot analysis with equal amounts of total RNA loaded on each lane showed a marked increase in the VCAM-1 mRNA transcript levels. **B:** Corresponding immunoblot analysis with a VCAM-1 antibody also showed an increase in the 110-kd protein at these time points.

expression of VCAM-1 in this model was preceded by the activation of the NF-κB/IκB system in these cells.[16] These findings prompted us to determine the time course of VCAM-1 and MCP-1 gene expression in SMCs after balloon injury and to correlate it with the activation of NF-κB and the adhesion of inflammatory cells.

Northern blot analysis performed on RNA isolated from the vessel wall demonstrated six- to sevenfold higher levels of VCAM-1 mRNA at both 3 and 7 days after balloon injury compared with normal vessels (Figure 4A). At 4 weeks after denudation, VCAM-1 mRNA expression returned to levels similar to control arteries. Western blotting confirmed this induction of VCAM-1 expression at the protein level. VCAM-1 protein was apparent as a 110-kd protein in normal carotid arteries with four- to fivefold higher levels present in the vessel wall at 3, 7, and 14 days (Figure 4B). In denuded vessels at 4 weeks, VCAM-1 protein levels were still two to three times above normal. To localize the sites of VCAM-1 mRNA expression within the vessel wall, we used *in situ* hybridization carried out on cross sections and on *en face* preparations. Although VCAM-1 expression was not detectable on cross sections of normal vessels, scattered positive SMCs were apparent within 4 hours after balloon injury (Figure 5A). By 3 days after injury, numerous SMCs in the tunica media expressed VCAM-1 (Figure 5B). Proliferating SMCs forming the luminal surface of the neointima at 8 days after denudation also showed high levels of expression (Figure 5C). Dramatic expression of VCAM-1 mRNA was apparent in most of these luminal SMCs at 4 weeks (Figure 5D) despite the fact that the vast majority of these cells were no longer replicating.[18] Using *en face in situ* hybridization, MCP-1 expression was also detectable at 8 days in many but not all luminal SMCs of the developing neointima (Figure 5E). At this time point, expression of MCP-1 appeared to be present mostly in SMCs that were not yet in contact with other SMCs, ie, individual SMCs that had just emerged from the media and cells along the edges of expanding clusters of SMCs. Numerous SMCs on the surface of chronically denuded vessels (4 to 6 weeks after denudation) also expressed MCP-1 mRNA (Figure 5F).

The *en face* approach allowed us to simultaneously correlate VCAM-1 or MCP-1 expression with the presence of inflammatory cells. Inflammatory cells were frequently found to adhere to SMCs on the luminal surface both at 8 days and 4 weeks after balloon injury, often occurring in clusters. Immunohistochemistry performed *en face* revealed that the vast majority of these inflammatory cells stained with the ED-1 antibody, which recognizes monocytes, macrophages, and dendritic cells (Figure 5, G and H). Very few adherent cells were CD5 positive, which is present on T and B cells (data not shown). To obtain additional quantitative data about the presence of inflammatory cells, immunoblotting was carried out with the ED-1 antibody. The antibody recognized a single band on reducing gels that was barely detectable in the normal vessel wall (Figure 6). The amounts of this monocyte/macrophage-derived protein increased with time after balloon injury and peaked at 4 weeks after denudation (Figure 6). Moreover, VCAM-1 is known to bind to $\alpha 4$ integrins on the surface of cells, and an $\alpha 4$ integrin antibody demonstrated the presence of inflammatory cells 2 and 4 weeks after injury as determined by immunohistochemistry (data not shown).

Discussion

NF-κB has been shown to play a role in regulating gene expression and proliferation in SMCs in culture,[13,14] but information about the role of NF-κB in SMCs in intact arteries is currently unavailable. The goals of the present study were to characterize the NF-κB/IκB system in vascular SMCs *in vivo*, provide evidence for NF-κB activation after arterial injury, and correlate it with SMC proliferation and the expression of the NF-κB-regulated genes VCAM-1 and MCP-1. We used the rat carotid artery balloon injury model and focused on time points when cells in the vessel wall were rapidly proliferating and compared them with the quiescent normal vessel wall. Using Western blot analysis, we demonstrated that all currently known Rel family members were present in the normal uninjured vessel wall. In response to balloon injury, the level of these proteins increased at times when SMCs were replicating and neointima formation occurred. Interestingly, transcriptional activation of NF-κB has recently been shown to be regulated by specific cyclin-dependent kinases.[33] Thus, the increase in NF-κB expression may reflect the induction of SMC replication in response to balloon injury. The magnitude of the increase in expression was different for each Rel family member. Whereas 2- to 3-fold increases were seen for p50 and p65, increases during intimal lesion formation were seen up to 15-fold for c-Rel and 8-fold for RelB. In normal vessels, p50 and p65 were readily detectable whereas c-Rel and RelB were less abundant. This expression pattern suggests that the Rel proteins that are most abundant in normal vessels (p50 and p65) may be important in

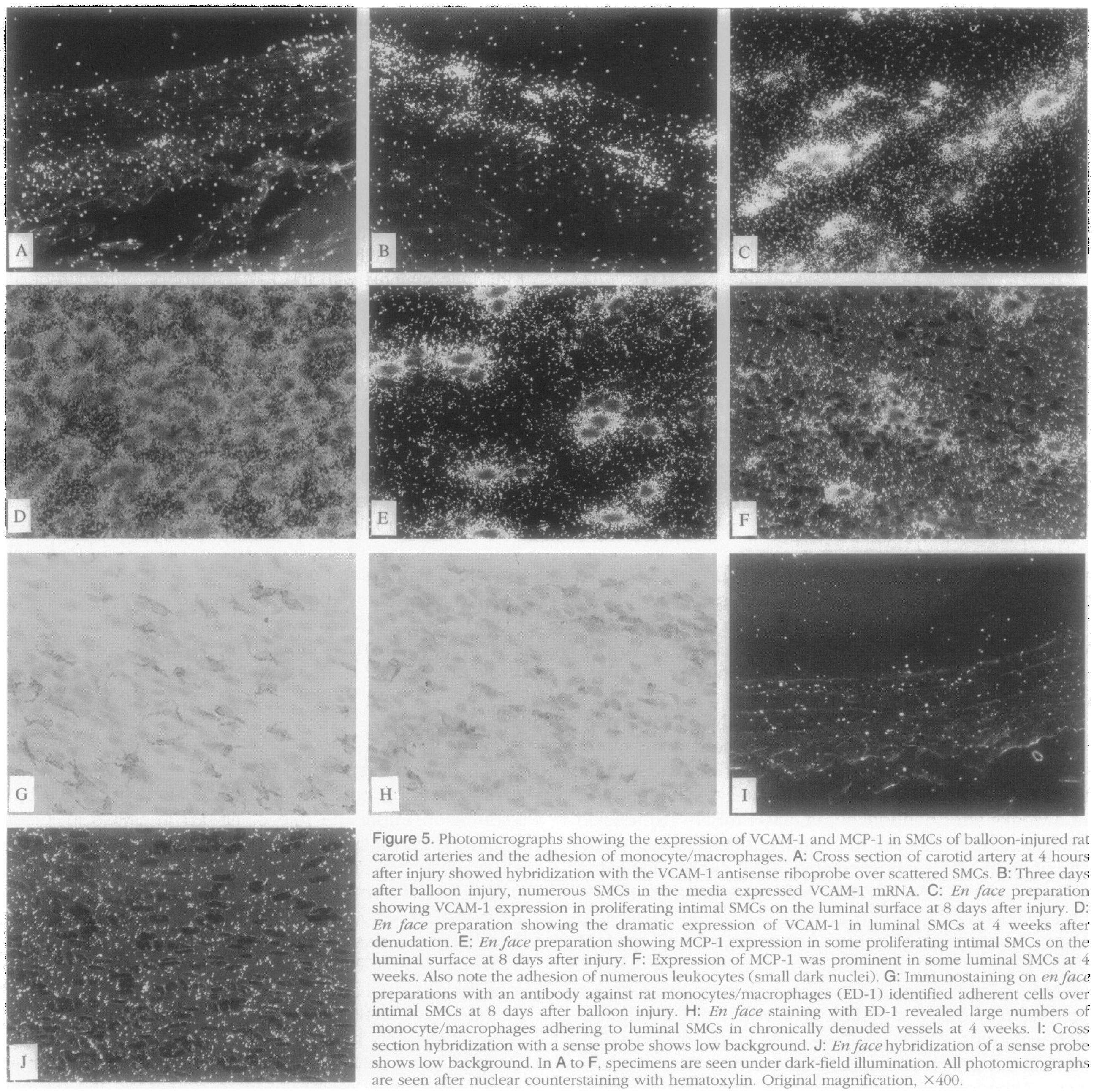

Figure 5. Photomicrographs showing the expression of VCAM-1 and MCP-1 in SMCs of balloon-injured rat carotid arteries and the adhesion of monocyte/macrophages. A: Cross section of carotid artery at 4 hours after injury showed hybridization with the VCAM-1 antisense riboprobe over scattered SMCs. B: Three days after balloon injury, numerous SMCs in the media expressed VCAM-1 mRNA. C: *En face* preparation showing VCAM-1 expression in proliferating intimal SMCs on the luminal surface at 8 days after injury. D: *En face* preparation showing the dramatic expression of VCAM-1 in luminal SMCs at 4 weeks after denudation. E: *En face* preparation showing MCP-1 expression in some proliferating intimal SMCs on the luminal surface at 8 days after injury. F: Expression of MCP-1 was prominent in some luminal SMCs at 4 weeks. Also note the adhesion of numerous leukocytes (small dark nuclei). G: Immunostaining on *en face* preparations with an antibody against rat monocytes/macrophages (ED-1) identified adherent cells over intimal SMCs at 8 days after balloon injury. H: *En face* staining with ED-1 revealed large numbers of monocyte/macrophages adhering to luminal SMCs in chronically denuded vessels at 4 weeks. I: Cross section hybridization with a sense probe shows low background. J: *En face* hybridization of a sense probe shows low background. In A to F, specimens are seen under dark-field illumination. All photomicrographs are seen after nuclear counterstaining with hematoxylin. Original magnification, ×400.

regulating immediate changes in gene expression whereas c-Rel and RelB proteins are newly synthesized and regulate later changes in gene expression. This has been demonstrated in T cells, where new c-Rel is synthesized after 4 hours of treatment with phorbol ester and a calcium ionophore.[34] At 4 weeks after injury, when SMCs have stopped proliferating, levels of Rel proteins decreased again with the exception of p52, which was highest at 4 weeks after injury.

Quantitating Rel protein expression by Western blotting does not conclusively demonstrate that these factors are modulating gene expression, as these molecules may be in an inactive form, nor can it determine which NF-κB complexes are important in this injury model. In general, inactive NF-κB is found in the cytoplasm bound to IκB, whereas activated NF-κB is found in the nuclei of cells. Brand et al detected activated p65 in human atherosclerotic lesions using an antibody recognizing the nuclear localization signal of p65; however, this antibody did not cross-react with rat p65 in our hands.[15] In addition, we were unsuccessful in isolating SMC nuclear and cytoplasmic proteins, as these cells are embedded in dense extracellular matrix of collagen and elastin from the vessel wall. Therefore, to address the issue of NF-κB activation, we examined the level of expression of the inhibitory proteins IκBα, IκBβ, and p105 after injury by Western blotting and the NF-κB binding activity by EMSA using whole-cell extracts. The levels of IκBα and IκBβ decreased immediately after injury, approximately 55 and 80%, respectively, and returned to normal within 3 to

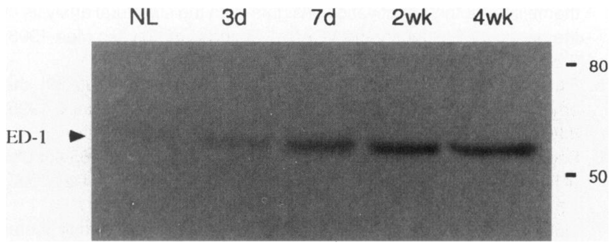

Figure 6. Western blot analysis of rat carotid artery extracts at various times after balloon injury probed with the ED-1 antibody (identifies monocyte/macrophages). Levels of inflammatory cells increased with time after balloon injury.

7 days after injury. The levels of p105, the precursor of p50, also decreased 58% after injury and remained reduced 3 days after injury, concomitant with an increase in p50 protein levels. The proteolytic cleavage of p105 may be an important regulatory step in increasing the levels of p50.

Constitutive NF-κB DNA binding activity was present in SMCs from normal carotid arteries and was dramatically reduced 1.5 hours after injury. It is not surprising that a low level of NF-κB activity is present in normal vessels, as constitutive NF-κB activity is present in cultured rat SMCs.[10] By 4 hours after injury, NF-κB binding activity was restored, and by 7 days after injury the NF-κB activity exceeded normal by fivefold. p65 and c-Rel are components of the induced complex and both contain a transcriptional activation domain that is absent from p50. Therefore, the uninhibited NF-κB binding activity that is present in normal vessels may not be as capable of activating transcription as the injury-induced NF-κB binding activity.

It is paradoxical that we detect constitutively active NF-κB in normal carotid arteries when IκB proteins are present and that IκB is restored in vessels when we detect an increase in activated NF-κB (3 to 7 days after injury). One possible explanation for this is that IκB protein turnover may be enhanced in these cells, therefore leading to the constant translocation of a small pool of NF-κB into the nucleus. This has been previously reported for IκBα in mature B cells,[35] which have constitutively activated NF-κB in the nucleus. The increase in NF-κB binding activity in 3- and 7-day-injured vessels may also be due to the increase in the ratio of NF-κB to IκB protein that we detected during this time period relative to normal vessels. An increase in NF-κB protein may lead to the saturation of IκB and allow a portion of the total pool of NF-κB to remain active. It is also possible that an as yet unidentified IκB may be regulating NF-κB in SMCs *in vivo* at these later times after injury. Interestingly, the level of constitutively activated NF-κB in normal vessels decreases within 1 to 1.5 hours after injury but begins to recover by 4 hours after injury. It is unlikely that IκBα, IκBβ, or p105 play a role in the inactivation of NF-κB 1.5 hours after injury, as the levels of all three of these proteins were also significantly reduced. Cell death as a consequence of the injury also cannot explain this finding as DNA loss after balloon injury in this model is less than 25%,[36] which is significantly less than the reduction seen for the inhibitory proteins and the NF-κB activity. Furthermore, the restoration of the NF-κB activity within 4 hours also argues against cell death causing the initial loss of NF-κB activity.

There is an apparent discrepancy between the expression levels of Rel proteins and NF-κB activation as determined by EMSA and supershift analysis. Whereas p50 and p65 protein levels increase only 2- to 3-fold, they seem to account for the majority of the NF-κB activity even though expression of RelB and c-rel was increased 8- to 15-fold. There are several potential explanations for this discrepancy. One, substantial levels of p50 and p65 are already present in the normal vessel wall whereas c-rel and RelB are almost undetectable in these vessels. Thus, the relative increase in c-rel and RelB protein levels is more pronounced. Second, the p50 and p65 turnover might be higher than the turnover for c-rel and RelB, which would be supported by the fact that the activated NF-κB complexes contain p50 and p65. Third, the influx of inflammatory cells may contribute disproportionately to the increase in c-rel, RelB, and p52 protein levels in the injured arterial wall, but these proteins may remain in an inactivated state. This would be supported by the fact that levels of c-Rel and p52 proteins in the chronically denuded vessels at 4 weeks were higher than in normal vessels and immunoreactivity for macrophages also peaked at 4 weeks after injury. Further clarification of these issues would require reagents that can specifically detect activated Rel proteins *in situ* in our *in vivo* model.

As significant numbers of macrophages are not seen in the vessel wall until 3 days after balloon denudation, the early decrease in IκB protein levels and the activation of NF-κB most likely reflect responses occurring in SMCs. At all later times, infiltrating macrophages are likely to contribute to the expression levels of IκB and Rel proteins. We have indeed observed expression of IκBα in macrophages by *in situ* hybridization (data not shown). However, the number of inflammatory cells present in the neointima at 2 weeks has been reported not to exceed 1%.[37] Thus, it is likely that even at later time points the majority of the NF-κB activity is derived from SMCs.

After endothelial wounding in rat arteries, we recently reported the induction of VCAM-1 expression within 4 hours at the wound edge, and this induction was preceded by nuclear translocation of p50 and p65.[16] In cultured SMCs, VCAM-1 expression has been shown to be mediated by NF-κB,[14] and it is likely that this also holds true for intact vessels. In this study, we sought to determine whether the time course of VCAM-1 gene expression coincided with the temporal activation of NF-κB and the decrease in IκB proteins. Indeed, by Northern blotting, VCAM-1 mRNA levels were six to seven times higher at 3 to 7 days after balloon injury while, at the same time, the levels of activated NF-κB were increased. Using *in situ* hybridization on cross sections, VCAM-1 mRNA was detectable in scattered SMCs as early as 4 hours after injury, and a newly activated NF-κB complex was induced by this time as determined by EMSA. Unlike endothelial cells along a wound edge, the stimulation of SMCs, and thus the induction of VCAM-1 mRNA, is not as synchronized, and this may also be true for NF-κB activation. This is supported by the fact that entry of SMCs into S-phase occurs over a period of several hours[38] after balloon denudation.

Another gene that we studied in response to balloon injury was MCP-1 (also known as *JE*), as its induction has also been shown to be mediated by NF-κB.[30] Taubman and co-workers[32] have reported that MCP-1 mRNA is induced in rabbit aortas within hours after balloon injury. In the present study, we observed a coordinated expression of VCAM-1 and MCP-1, and in denuded arteries these genes seemed to be expressed only by SMCs. Northern blot analysis showed VCAM-1 mRNA levels returned to normal at 4 weeks after injury concomitant with the reduced level of NF-κB activation. However, *in situ* hybridization, which identifies the mRNA of a single cell, revealed that luminal SMCs in chronically denuded vessels are highly activated, expressing high levels of VCAM-1 and MCP-1. We do not detect elevated levels of NF-κB activation in chronically denuded vessels possibly due to the fact that only a small portion of the SMCs are activated.

With *en face* preparations, we were able to link the expression of VCAM-1 and MCP-1 to the adhesion of inflammatory cells, the majority of which were monocyte/macrophages. Immunoblotting also demonstrated that the increase in macrophage accumulation in the vessel wall occurred in parallel with the increase in VCAM-1 expression. During mammalian muscle development, VCAM-1 and its corresponding receptor VLA-4 ($\alpha 4\beta 1$ integrin) are expressed at sites of secondary myogenesis. To determine whether a VCAM-1/VLA-4-mediated interaction occurred among SMCs in the injured vessel wall, we carried out immunohistochemistry with an antibody against the rat $\alpha 4$ integrin and found that it was expressed only on the adherent leukocytes (data not shown).

In summary, the present study investigated the NF-κB/IκB system in a vascular injury model. NF-κB activity was induced and the protein expression of NF-κB family members was up-regulated at times of rapid SMC proliferation and neointima formation. These changes in the NF-κB/IκB system coincided with an increase in cell proliferation and the expression of VCAM-1 and MCP-1, which was accompanied by the influx of monocyte/macrophages. These findings may be relevant to human vascular pathologies associated with SMC proliferation such as restenosis after angioplasty and atherosclerosis. Together, our data suggest that the NF-κB/IκB system is activated after vascular injury and plays a role in regulating genes that control SMC activation and proliferation and consequently vascular inflammation.

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